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# Changes in the Proliferation of the Subventricular Zone Neural Stem Cell Pool throughout Aging in the Murine Brain

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# **Changes in the Proliferation of the Subventricular Zone Neural Stem Cell Pool throughout Aging in the Murine Brain**

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## **TABLE OF CONTENTS**

### **I. ABSTRACT**

### **II. INTRODUCTION & BACKGROUND**

- A. Historical Overview of Adult Neurogenesis
- B. Cytoarchitecture of the Subventricular Zone
- C. SVZ B1 Astrocytes are the SVZ Neural Stem Cell
  - 1. Ependymal Cells versus Astrocytes
  - 2. Non-specificity of Molecular Markers
- D. The Aging SVZ Neural Stem Cell Niche
  - 1. Embryonic to Neonatal Brain Development
  - 2. Gross and Cytoarchitectural Changes throughout Aging
  - 3. Age-related Decline in Neurogenesis

### **III. EXPERIMENTAL PROCEDURES**

- A. Animals
- B. Wholemount Preparations
- C. Immunohistochemistry
- D. BrdU Studies
- E. Lazarus Retroviral Studies

### **IV. RESULTS**

- A. Neural Stem Cell Marker Specificity
- B. Ventricle-Contacting Astrocytes are Neural Stem Cells
- C. Detection of Neural Stem Cells for pH3

### **V. DISCUSSION**

### **VI. ACKNOWLEDGEMENTS**

### **VII. REFERENCES**

## I. ABSTRACT

In the 1990s, the dogma that new neurons could not be produced in the adult mammalian brain was laid to rest when two regions of the brain were discovered to participate in neurogenesis. Of these two regions, the subventricular zone (SVZ) of the lateral walls of the lateral ventricle demonstrates the largest area of neurogenesis within the adult mammalian encephalon. The production of new neurons from this region is attributed to the proliferation and differentiation of SVZ neural stem cells (NSCs).

Distinguishing what cell in the SVZ is the NSC has been a tumultuous journey for researchers. Currently, B1 astrocytes are widely believed to be the NSCs of the SVZ as these cells have been documented to self-renew, be quiescent, and multipotent. B1 astrocytes also share a lineage with embryonic NSCs, radial glia (RG). It has been hypothesized that as RG differentiate into ependymal cells, their apical surface expands, constricting the remaining RG to small clusters surrounded by these newly-formed ependymal cells. This pinwheel organization, apparent during the embryonic stages of life, persists in adulthood with B1 astrocytes replacing RG in this cytoarchitectural design. These B1 astrocytes are morphologically reminiscent of RG, as both maintain an apical contact in the ventricle and a basal contact on blood vessels. The shared lineage and structure between RG and B1 astrocytes help in affirming B1 astrocytes as SVZ NSCs.

Prospective identification of SVZ NSCs is critical to the advancement of the field of neural stem cell biology and the creation of possible stem cell therapies targeted to the nervous system. Using molecular markers against certain structures of a cell of interest has been a tool widely used in the field. However, researchers must be cautious with this method of identification since many markers can localize on structures of multiple cell types and depict an inaccurate picture of cellular modalities. To ameliorate this issue, several techniques should be used in conjunction with immunohistochemistry for proper identification. In a novel approach, this study identifies NSCs using both immunohistochemistry and the NSC ventricle-contacting morphology visualized in wholemount preparations. Once properly

distinguished, NSCs can then be appropriately observed participating in various biological processes; of interest to this study is the process of aging.

Previous studies have noted a decrease in neurogenesis as a consequence of decreased SVZ proliferation throughout aging. It is still not known whether this decline in output is attributed to a decrease in NSC number and/or activity. This study will quantify the percentage of proliferating NSCs at various ages of adult murine brain development in order to elucidate a possible correlation between NSC division kinetics and the declining neurogenic output witnessed with aging.

## **II. INTRODUCTION & BACKGROUND**

### **A. Historical Overview of Adult Neurogenesis**

Throughout a majority of the 19th and 20th centuries, the central dogma that new neurons cannot be produced in the adult mammalian brain dominated the field of neurobiology. It was widely believed that neurogenesis only occurred during embryogenesis, but after the postnatal completion of brain development all neuronal networks were permanently set in place (Colucci-D'Amato *et al.*, 2006).

The first documented attempt to unravel this central dogma was by Allen in 1912 (Chojnacki *et al.*, 2009). Using thionin and iron-alum-haematoxylin to stain cells undergoing mitosis, Allen was able to visualize mitotically active cells in the adult mammalian rat brain, with the greatest and longest persisting postnatal mitotic activity on the lateral walls of the lateral ventricles (Allen, 1912). Thymidine is a chemical compound integrated into the DNA strand during DNA synthesis. Its incorporation into a cell indicates that the cell has entered the cell cycle to become a proliferating cell. Tritiated thymidine (<sup>3</sup>H- thymidine) is used to label proliferating cells and subsequently highlight the amount of cellular proliferation in a region of interest (Chojnacki *et al.*, 2009; Dolbeare, 1995). However, bromodeoxyuridine (BrdU) is a more popular compound because of its reduced toxicity, greater sensitivity, and its ability to be visualized using immunohistochemistry (Dolbeare, 1995; Colucci-D'Amato

*et al.*, 2006). Both compounds are also utilized in label-retention assays to reveal the quiescent quality of cells (Dolbeare, 1995; Chojnacki *et al.*, 2009).

In 1958, Messier *et al.* were able to pinpoint clusters of  $^3\text{H}$ -thymidine-labeled cells to regions beneath the ependymal layer of the lateral ventricles in adult mice (Chojnacki *et al.*, 2009; Messier *et al.*, 1958). Although these experiments highlighted active proliferation in the adult mammalian brain, they did not provide evidence for the production of new neurons resulting from this proliferation.

During the 1960s and 1970s the experiments of Joseph Altman and Michel Kaplan using  $^3\text{H}$ -thymidine and electron microscopy demonstrated that the adult mammalian brain does generate new neurons (Colucci-D'Amato *et al.*, 2006; Chojnacki *et al.*, 2009). In the 1980s, Nottenbohm and collaborators established the occurrence of neurogenesis in song birds and the importance of this phenomenon to learning songs; illustrating a functional consequence to neurogenesis (Colucci-D'Amato *et al.*, 2006; Nottenbohm, 1984; Alvarez-Buylla *et al.*, 1988). Despite the data, it was not until the 1990s that the scientific community began to accept the idea of adult mammalian neurogenesis.

The neurosphere assay has been a method extensively employed to demonstrate the self-renewal and differentiation potential of NSCs. In the assay, dissociated nerve cells are induced to proliferate in the presence of a mitogen *in vitro*. The proliferation generates a sphere-cluster of undifferentiated cells, the neurosphere. The neurosphere contains the neural stem cells as well as neural precursors. In certain culture conditions where mitogens are absent, neurospheres or their dissociates will generate neurons, astrocytes, and oligodendrocytes (Chojnacki *et al.*, 2009).

It was during the 1990s Weiss and Reynolds conducted a neurosphere assay for isolated cells from the adult mouse striatum. Practically all the cells comprising of the neurospheres were positive for nestin, a marker for NSCs. Cells of the neurosphere were dissociated into single cells. These dissociates were re-plated to self-renew and to differentiate into neurons and astrocytes. Thus, Weiss and Reynolds had isolated adult mammalian neural stem cells (Colucci-D'Amato *et al.*, 2006; Chojnacki *et al.*,

2009; Reynolds *et al.* 1992). Following in the steps of Weiss and Reynolds, Lois and Alvarez-Buylla established that cells from the subventricular zone (SVZ) could also generate neurons and astrocytes (Lois *et al.*, 1993). The retroviral studies of Luskin (Luskin, 1993) and the BrdU labeling of SVZ cells by Corotto (Corotto *et al.*, 1993) manifested the neurogenic and neural migratory properties of the SVZ *in vivo* (Chojnacki *et al.*, 2009).

As a result of the previous scientists and many others, two major areas of neurogenesis within the adult mammalian encephalon have been identified (Colucci-D'Amato *et al.*, 2006; Conover *et al.*, 2008, 2010; Doetsch *et al.*, 2003). One site is the subgranular zone (SGZ) of dentate gyrus in the hippocampus. The other area is the SVZ located along the lateral walls of the lateral ventricles. It is in these regions where resident adult NSCs self-renew and proliferate until a final differentiated cell type is generated (Colucci-D'Amato *et al.*, 2006; Conover *et al.*, 2008, 2010; Doetsch *et al.*, 2003).

## **B. Cytoarchitecture of the Subventricular Zone**

The SVZ contains the largest capacity for neurogenesis in the adult mammalian brain. Four cell types contribute to the architecture of the SVZ—neuroblasts (type A cells), ependymal cells (type E cells), transit-amplifying progenitor cells (type C cells), and astrocytes (type B cells) (Conover *et al.*, 2008, 2010; Doetsch *et al.*, 1997, 2003). Ependymal cells are multi-ciliated epithelial cells that tightly line the ventricle (Lenington *et al.*, 2003; Conover and Shook, 2010). Beneath this layer of cells rests the active cells of the SVZ.

Evidence has suggested that astrocytes within the SVZ can be divided into three layers (Shen *et al.*, 2008). The basal astrocytes near the striatal border, 20µm below the ependymal surface, possess multiple processes characteristic of mature astrocytes (Shen *et al.*, 2008). Above the basal astrocytes are tangential astrocytes located 10µm below the ependymal layer with only one or two processes. These astrocytes run along the anterior-posterior direction, the same direction as the neuroblasts (Shen *et al.*, 2008). These tangential and basal astrocytes make up the niche astrocytes (B2) that ensheath

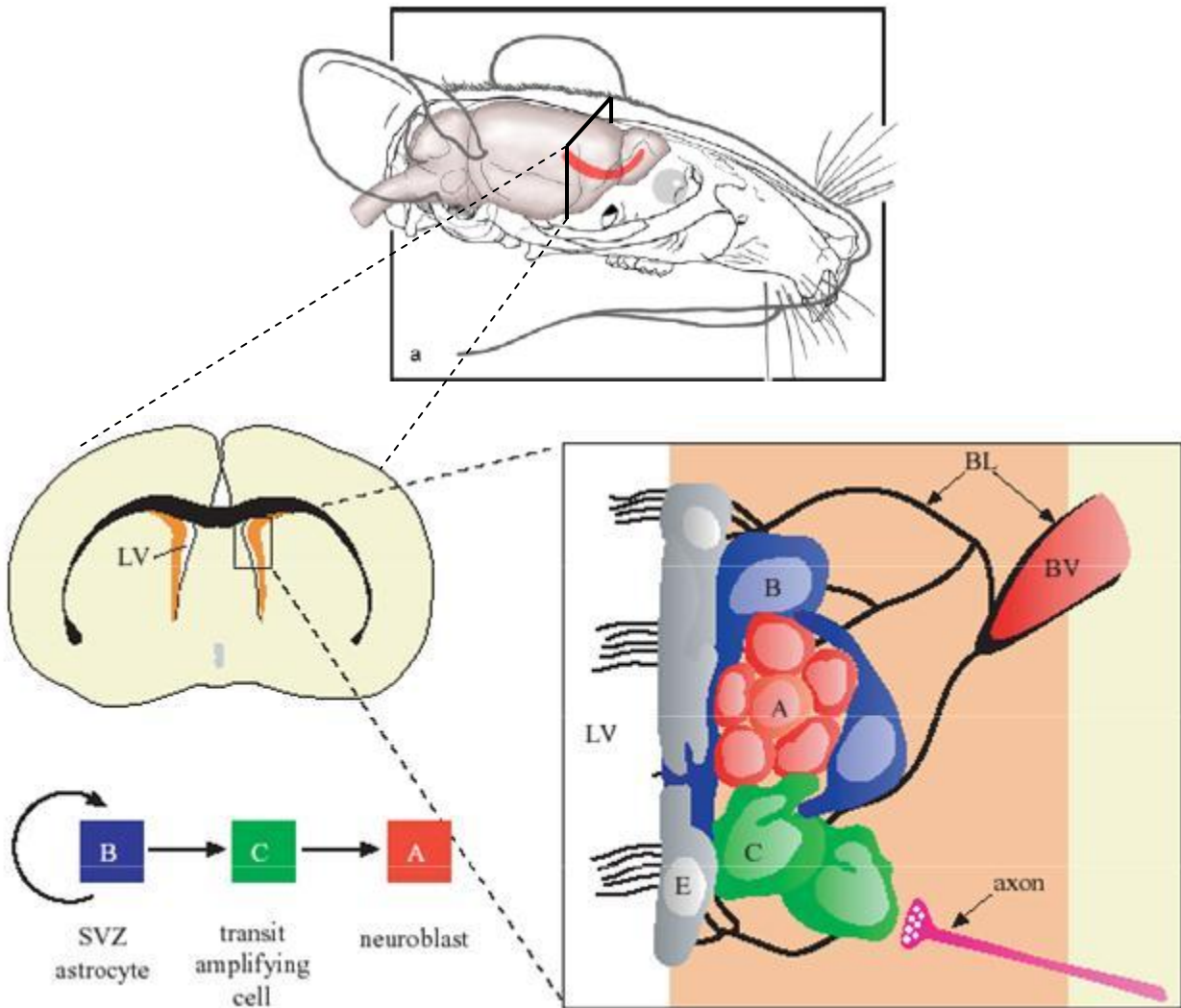
neuroblasts as they migrate in chains along the rostral migratory stream (RMS) (Lois *et al.*, 1996; Shen *et al.*, 2008). The apical astrocytes (B1), within 5µm of the ependyma, present a flattened morphology reminiscent of primitive neuroepithelial cells. B1 astrocytes contact the ventricle intercalating into the ependymal layer (Doetsch *et al.*, 1997, 1999b; Shen *et al.*, 2008). The nuclei of these apical cells can range from near the ependyma all the way towards the blood vessels within the region, where Shen *et al.* found many of these nuclei (Shen *et al.*, 2008). The location of the nuclei can be indicative of where astrocytes are during the cell cycle (Mirzadeh *et al.*, 2008).

The neural stem cells (NSCs) of the SVZ are apical astrocytes that maintain an apical process contacting the ventricle and a basal process with endfeet contacting a blood vessel (Kriegstein *et al.*, 2009; Mirzadeh *et al.*, 2008; Shen *et al.*, 2008; Tavazoie *et al.*, 2008). The apical process contains one primary cilium extending into the cerebral spinal fluid (CSF) from the top of the apical process (Doetsch *et al.*, 1997, 1999b). NSCs generate type C progenitors that rapidly divide to give rise to neuroblasts and glial cell populations. Neuroblasts migrate in chains along the rostral migratory stream (RMS) and differentiate into periglomerular interneurons or granule cells in the olfactory bulb (Lois *et al.*, 1994). Intermingled throughout the niche are basal lamina from blood vessels and, at the basal layer of the niche, blood vessels which allows these cell types access to diffusible factors of the blood (Shen *et al.*, 2008; Tavazoie *et al.*, 2008)

In an *en face* view of the ventricular surface, Mirzadeh *et al.* identified pinwheel structures of cells largely located in the neurogenic zone of the lateral ventricles. These pinwheels consist of E1, E2, and B1 cell types (Chojnacki *et al.*, 2009; Mirzadeh *et al.*, 2008). E1 cells are the most prominent of the apical surface cells both in number and in apical surface area. These cells contain tufts of long cilia with small basal bodies (Mirzadeh *et al.*, 2008). E2 cells are bi-ciliated with cilia longer than E1 cells and they contain large, lobular basal bodies (Mirzadeh *et al.*, 2008). B1 cells are apical astrocytes with short single cilia and, of the three cells, possess the smallest apical surface area (Doetsch *et al.*, 1999b; Mirzadeh *et*



*al.*, 2008). The larger apical surfaces of the multi-ciliated E1 and E2 ependymal cells surround generally small pockets of singly ciliated B1 astrocytes (1-2 B1 cells) producing pinwheel structures throughout the lateral wall of the lateral ventricle. However, the anterior-ventral (AV) and posterior-dorsal (PD) areas of the lateral walls of the lateral ventricle maintain pinwheels with B1 clusters exceeding greater than 10 B1 cells (Mirzadeh *et al.*, 2008).



Adapted from Lenington *et al.* (2003) and Riquelme *et al.* (2008)

**Figure 1 Cytoarchitecture of the Subventricular Zone.** Four major cell types compose the subventricular zone (in orange) neuroblasts (A), astrocytes (B), transit amplifying cell (C), and ependymal cells (E). The subpopulation of the astrocytes contacting the cerebral spinal fluid (CSF) and expressing a single cilium is thought to be the stem cells of the region. These astrocytes are also known as B1 astrocytes. Astrocytes of this nature self-renew and give rise to type C cells which then give rise to neuroblasts

(Conover *et al.*, 2008; Doetsch *et al.*, 2003). These migratory neuroblasts move along the rostral migratory stream to be incorporated into the olfactory bulb. In the midst of these SVZ cell types are basal lamina (BL) originating from nearby blood vessels (BV).

### **C. SVZ B1 Astrocytes are the SVZ Neural Stem Cell**

#### *1. Ependymal Cells versus Astrocytes*

To be an adult stem cell, there are several qualities that a cell must possess. The cell must slowly divide—expressing quiescence, be multipotent, and be able to self renew. When given a particular signal, the cell, in our case the SVZ NSC, is reprogrammed to enter the cell cycle or transform itself to another cell type down the lineage (Conover and Shook, 2010 and Kriegstein *et al.*, 2009). This behavior mimics that of RG in the early mammalian brain (Kriegstein *et al.*, 2009). If the NSC enters the cell cycle, it divides asymmetrically so that one daughter cell remains a NSC and the other cell gives rise to a progenitor cell, type C cell or the neural intermediate progenitor cell (nIPC) (Kriegstein *et al.*, 2009). The progenitor cell divides and gives rise to more lineage specific progenitors, neuroblasts. Neuroblasts then give rise to a final differentiated cell type, interneurons as stated earlier.

During the mid-late 1990s, scientists were trying to understand which cell type within the SVZ were the NSCs of the region--ependymal cells or astrocytes. In 1999, Johansson *et al.* published the first paper regarding the identification of SVZ NSCs stating that ependymal cells were the NSC in the adult mammalian brain (Chojnacki *et al.*, 2009; Johansson *et al.*, 1999). The group stated that the ependymal cells must be the NSCs because of their ventricular zone location relative to type C cells, increased nestin (an intermediate filament protein with widespread expression during development and diminished expression during adulthood, traditionally found in NSCs) staining from the ependymal layer into the subventricular zone after injury in the spinal cord, and Dil expressing cells (of the ependyma) were able to generate neurospheres (Johansson *et al.*, 1999). That same year, Doetsch *et al.* published their paper saying that the astrocytes were the SVZ NSCs (Chojnacki *et al.*, 2009; Doetsch *et al.*, 1999a). Using retroviruses encoding for green fluorescent protein (GFP), label-retention assays of proliferation

markers, and the NSA; Doetsch *et al.* demonstrated that SVZ astrocytes, and not ependymal cells, generated type C cells and neuroblasts (illustrating multipotency) and that the astrocytes, not ependymal cells, slowly divided (quiescence) and had the ability to self-renew (Doetsch *et al.*, 1999a).

In 2008, Mirzadeh *et al.* went a step further and identified an apical surface pinwheel organization unique to the neurogenic regions of the SVZ consisting of ependymal cells and ventricle contacting B1 astrocytes. Mirzadeh noted how these B1 astrocytes retained glial fibrillary acidic protein (GFAP) positive contacts with blood vessels and the ventricle, sharing these morphological components with embryonic RG. An adenovirus expressing Cre under the GFAP promoter, located in astrocytes was injected into a lateral ventricle of a Z/EG double reporter mouse. The Z/EG (lacZ/EGFP) mouse has two reporters lacZ and enhanced green fluorescent protein (EGFP). In the presence of the Cre enzyme, expressed by a virus in this case, the lacZ gene is excised activating the expression of EGFP (Novak *et al.*, 2000). The fact that the virus was injected into the ventricle only allowed astrocytes that had a ventricular contact (B1 astrocytes) to take up the virus through epithelial transport mechanisms since ependymal cells do not possess the GFAP promoter (Mirzadeh *et al.*, 2008). As proliferation occurs, cells that are progeny to B1 astrocytes will retain the virus and express GFP (Mirzadeh *et al.*, 2008; Novak *et al.*, 2000). On the ipsilateral lateral wall from the injection site, many ventricle-contacting B1 astrocytes were labeled (Mirzadeh *et al.*, 2008). Thirty days later, many neuroblasts and neurons labeled with GFP were present in the olfactory bulb. And most notably, the same was true for the contralateral lateral wall, although to a lesser extent (Mirzadeh *et al.*, 2008). B1 astrocytes were also isolated from the lateral wall and differentiated *in vitro* into neurons, oligodendrocytes, and astrocytes indicating the multipotency of B1 astrocytes (Mirzadeh *et al.*, 2008). With this work, it is evident why some researchers initially believed that ependymal cells, the only cells at the time that researchers knew contacted the ventricle, were SVZ NSCs. The ventricular cells that had expressed traditional NSCs markers, such as nestin, were B1 ventricle contacting astrocytes and not ependymal cells.

## 2. Non-specificity of Molecular Markers

Research throughout the years has investigated various qualities of SVZ NSCs using cell cycle markers, intracellular proteins, viral infections with viruses encoding reporter genes, and, more recently, glycoconjugates on the cell surface and transgenic mice (Chojnacki *et al.*, 2009).

Of all the tools utilized in the field, molecular markers against certain structures of a cell of interest have been a tool widely used in the field of neurobiology. However, when using this method of identification, researchers must be cautious since many of these markers can localize on structures of various cell types and depict inaccuracies in cellular modalities (Conover and Shook, 2010). To ameliorate this issue, multiple methods should be used in conjunction with immunohistochemistry for proper identification.

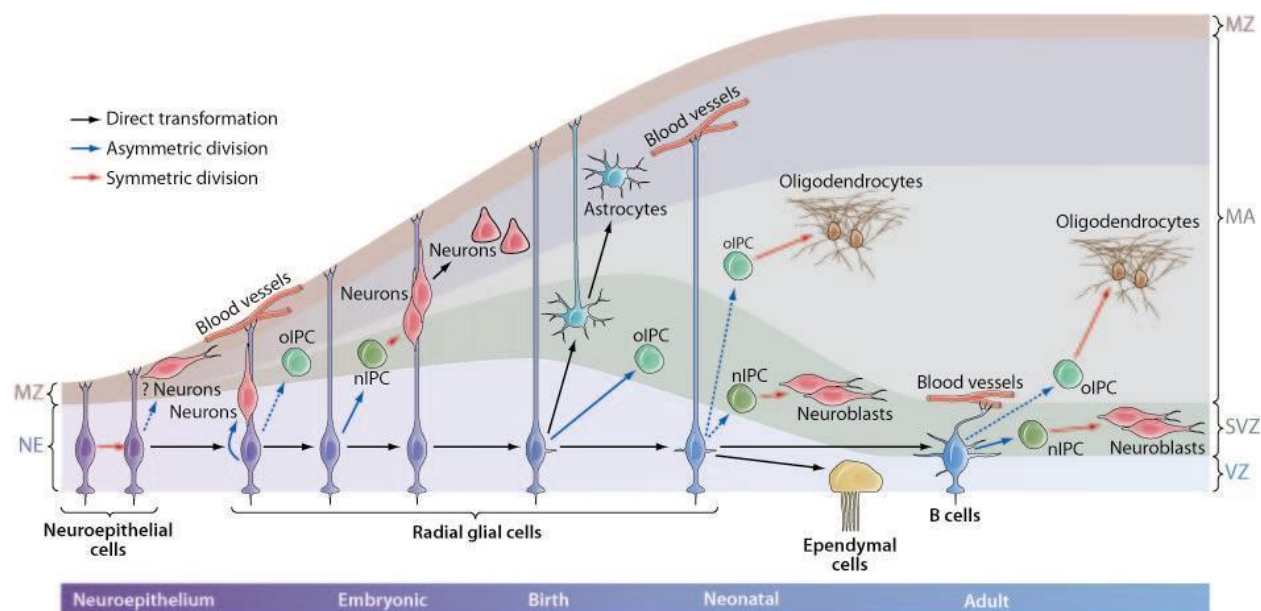
Phospho-histone H3 (pH3), Ki-67, and minichromosome maintenance 2 (MCM2) label proliferative cell nuclei, and for this study, aided the visualization of individual NSC nuclei. However, because these markers are not restricted to NSC nuclei (see figure 4 for examples of molecular marker non-specificity), an added morphological component was used to discriminate SVZ NSC astrocytes. This morphological component consists of using wholemount preparations of mouse brains to view and trace the glial fibrillary acidic protein (GFAP) positive apical processes of NSCs to the nuclei of SVZ NSCs. In order to distinguish the ependymal cell layer, beta-catenin and gamma-tubulin marked locations of cellular junctions and the cilia of ventricular cells, predominantly ependymal cells, respectively.

### **D. The Aging Subventricular Zone Neural Stem Cell Niche**

#### *1. Embryonic to Neonatal Brain Development*

In early development, neuroepithelium is the fertile ground for the multitude of cells of the nervous system. Neuroepithelial (NE) cells maintain a single process with a primary cilium contacting the ventricle and another process maintained in the marginal zone on the pial surface. After some time going through symmetric division to generate more NE cells, these cells transform themselves into

primitive radial glia (RG). RG maintain the original contact of NE cells with the ventricle but also begin to contact blood vessels within the marginal zone even as the brain epithelium thickens as a result of rapid proliferation. In the embryonic and neonatal brains, RG give rise to progenitor cells that become neurons and oligodendrocytes as well as maintaining the ability to self-renew through asymmetric divisions. RG cells mature to sprout additional processes located near the nuclei in the neonatal brain. These mature RG transform into ependymal cells and SVZ astrocytic NSCs. The NSCs retain the RG function of producing neurons and possibly oligodendrocytes through asymmetric division, where one of the daughter cells is a NSC and the other a progenitor cell. (Kriegstein *et al.*, 2009)



**AR** Kriegstein A, Alvarez-Buylla A. 2009.  
Annu. Rev. Neurosci. 32:149–84

**Figure 2 Radial Glia Lineage.** Astrocytes (B cells) exhibit many morphological and functional characteristics similar to RG which have been established as the neural stem cell of the embryonic and early postnatal brain. These similarities are a result of astrocytes sharing part in the lineage of radial glial cells; reinforcing the notion of astrocytes being the neural stem cells of the adult SVZ. The figure tracks the lineage of the SVZ astrocytes through a series of divisions and neuron and oligodendrocyte intermediate progenitor cells (nIPCs and oIPCs respectively). Note how adult astrocytes maintain a basal contact with blood vessels and an apical process in the ventricular zone (VZ). As with RG cells of the embryonic brain the apical process juts one primary cilium into the ventricle. Evidence supporting divisions are represented with solid lines. Dashed lines represent hypothetical divisions.

## 2. Gross and Cytoarchitectural Changes throughout Aging

With increased age in the postnatal mouse, many changes occur in the SVZ stem cell niche. On a global scale, the ventral aspect of the lateral ventricle begins to stenose restricting the functional niche to the dorsolateral region of the lateral ventricle (Conover and Shook, 2010; Luo *et al.*, 2006). An enlarged ventricular cavity produces a thinning ependyma, which now is composed of both ependymal-like cells as a result of ependymogenesis from NSCs and ependymal cells (Conover and Shook, 2010; Luo *et al.*, 2008). These ependymal-like cells are NSCs that have incorporated into the ependymal monolayer exhibiting adherens junctions and a loss of apical processes (Luo *et al.*, 2008; Mirzadeh *et al.*, 2008). This process has exclusively been observed in elderly mice (~2yr); however, with a moderate loss of ependyma in young mice (~3mo), ependymal cell replacement is also noted (Luo *et al.*, 2008).

### 3. Age-related Decline in Neurogenesis

When comparing young (2-4mo) and elderly (22-24mo) mice many researchers have noted a 50% decline in neurogenesis (Ahlenius *et al.*, 2009; Luo *et al.*, 2006; Tropepe *et al.*, 1997). This decrease in SVZ proliferation has been noted to manifest itself as a reduction in the amount of interneurons incorporated into the olfactory bulb (Enwere *et al.*, 2004). As a result, elderly mice are incapable of fine odor discernment between two similar odors, while young mice enjoy both fine and coarse odor discrimination (Enwere *et al.*, 2004; Sahin *et al.*, 2010).

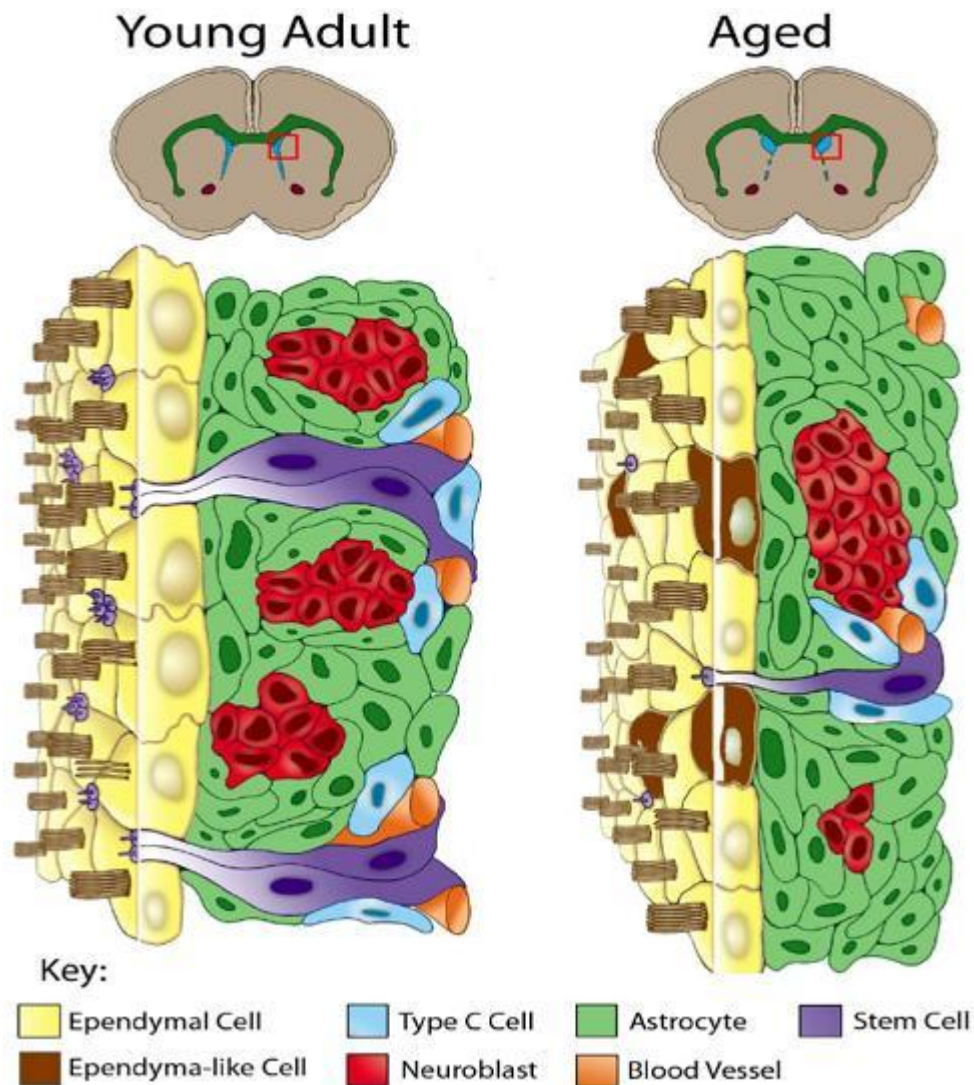
Several mechanisms have been implicated in the diminishing neurogenic output of the aging SVZ. Growth factors signaling levels, including transforming growth factor-alpha (TGF-alpha) and epidermal growth factor (EGF), are decreased within the elderly SVZ compared to the young SVZ (Enwere *et al.*, 2004; Tropepe *et al.*, 1997). Senescence of progenitor cells has also been observed, with 1.5-fold increase by mid-age (Luo *et al.*, 2006) and a 2-fold increase in elderly mice (Tropepe *et al.*, 1997) in the number of cells that stop dividing compared to young mice (Conover and Shook, 2010). Apoptosis is another factor, demonstrated in the study by Luo *et al.* in 2006 where neuroblasts experienced more apoptosis in the mid-aged mouse than the young. At the same time, fewer GFAP+ astrocytes were

apoptotic in the mid-aged mouse than the young. Moreover, the total number of astrocytes throughout aging remained relatively stable, only exhibiting a slight downward trend. These results (Luo *et al.*, 2006) suggest a bi-potential role for SVZ NSCs to participate in ependymal cell replacement and neurogenesis (Luo *et al.*, 2006).

All studies exploring the preceding mechanisms are in consensus regarding decreased progenitor proliferation and reduced numbers of progenitor cells present throughout the aging SVZ. However, the activity of NSCs remains unclear.

In 2009, Ahlenius *et al.* examined the effect aging has on NSCs and their contribution, or lack thereof, to the waning levels of neurogenesis. Using BrdU and doublecortin (Dcx) staining, the study was able to conclude a 58% decrease in the proliferative fraction of neuroblasts in elderly mice compared to young mice (Ahlenius *et al.*, 2009). In the case of the NSC the transcription factor sex determining region Y-box 2 (Sox2) and BrdU co-labeling indicated a 38% decrease in proliferative NSCs (Ahlenius *et al.*, 2009). Despite the lower numbers, active progenitor cells of elderly mice still maintained a capacity for proliferation and differentiation comparable to that witnessed in embryonic mice in the presence of exogenous cues *in vitro* (Ahlenius *et al.*, 2009; Luo *et al.*, 2006).

One issue with the preceding studies was that molecular markers were the only means employed to identify NSCs—an indefinite manner to identify cells. Therefore, this present study will look to use both molecular markers and wholemount preparations of the lateral ventricle to visualize ventricle contacting astrocytes, the NSCs, within the SVZ. The study will then quantify the proliferative fraction of NSCs throughout aging.



**Figure 3 The young and aged SVZ niche.** In the young mouse, clusters of astrocytic stem cells contact the cerebral spinal fluid (CSF) ejecting a primary cilium into the ventricle and surrounded by ependymal cells in a pinwheel structure. These stem cells also contact blood vessels in the basal aspect of the SVZ. In the aged mouse, there are fewer stem cells. When found, stem cells are not in the clusters evident in young mice. Proliferating astrocytes incorporate into the ependymal cell layer obtaining ependymal attributes and becoming ependymal-like cells (Luo and Shook, 2008). On the gross anatomical scale, the ventricle enlarges and stenosis occurs along the ventral portion of the SVZ.

### III. EXPERIMENTAL PROCEDURES

#### A. Animals

CD1 mice breeders were purchased from Charles River Laboratories and an in-house colony was generated and aged in our vivarium. In the following experiments, 3-month-old male mice were



designated young adult; 1-year-old, middle-aged; and 2-year-old, elderly. Animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut and conform to National Institutes of Health guidelines.

## **B. Wholemount Preparations**

Mice were anesthetized with 2.5% avertin (Sigma), used at .025mg/ kg of body weight, and then perfused transcardially by gravity perfusion with 0.9% saline. Brains were removed and dissected to reveal the lateral ventricles. These wholemounts were fixed overnight in 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) in phosphate buffered saline (PBS). The next day, the wholemounts were washed in PBS three times for 15 minutes, permeabilized and blocked in 1% Triton X-100 (Sigma, St. Louis, MO) and 10% horse serum (Invitrogen) in PBS for 1 hour. After staining procedures were completed, wholemounts were trimmed to include the part of the lateral ventricle that comprised of the SVZ. These wholemounts were then mounted onto slides, and coverslipped using aquapolymount (Polysciences, Warrington, PA). Wholemounts were imaged using a Leica TCS SP2 confocal laser-scan microscope (Bannockburn, IL) and the software Stereo Investigator (Microbrightfield, Inc, Williston, VT). Cells were also counted using Stereo Investigator.

## **C. Immunohistochemistry**

Whole mounts were incubated with the following primary antibodies in different combinations overnight at 4°C: beta-catenin, gamma-tubulin, GFAP, pH3, Ki67, and MCM2. Wholemounts were washed in PBS three times for 5 minutes, incubated with the appropriate Alexa Fluor dye-conjugated secondary antibodies (Molecular Probes, Eugene, OR) for 1 hour, and washed three times for 5 minutes in PBS.

## **D. BrdU Studies**

A series of 10 BrdU injections were given to 3-month-old animals over 10 days, 1 injection per day. After 10 days, mice were sacrificed, brains were harvested, and wholemount preparations were

made all in accordance with the aforementioned wholemount preparation protocol. Wholemounts were stained for beta-catenin, gamma-tubulin, and GFAP.

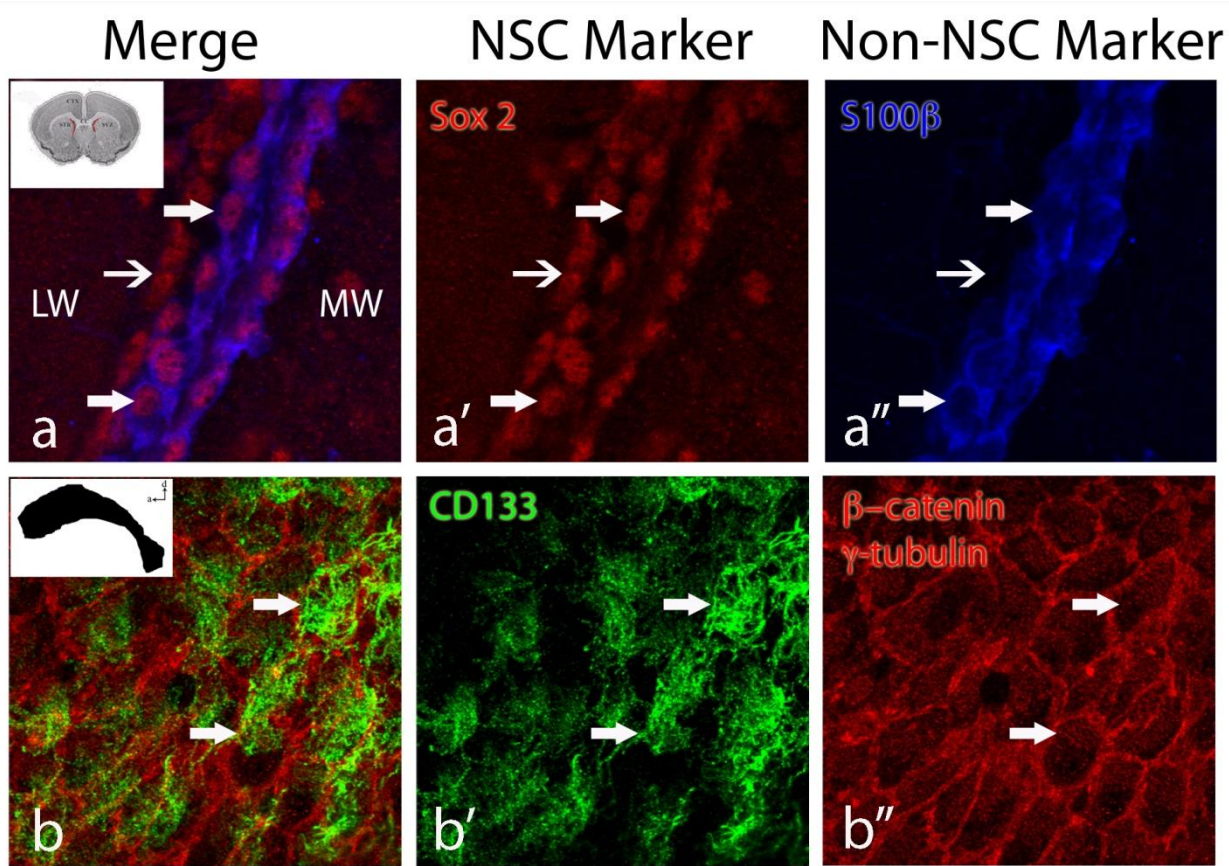
#### **E. Lazarus Retroviral Studies**

Lazarus (LZRS) retrovirus was injected into the ventricle of 3-month-old animals. Once injected into the ventricle, the virus was taken up by dividing cells of the ventricular surface. 24-hours after the injection, wholemount preparations were extracted from the mice in accordance with the aforementioned wholemount preparation protocol. After which, the preparations were stained for beta-catenin.

### **IV. RESULTS**

#### **A. Neural Stem Cell Marker Specificity**

In order to examine the specificity of molecular markers, NSC markers were co-stained with non-NSC markers in coronal sections and wholemount preparations. For the coronal sections, the subventricular zone was immunostained with Sox2, the NSC marker, and S100-beta, a calcium-binding protein found in the cytoplasm of SVZ ependymal cells (Raponi et al., 2007) (figure 4a-a''). In the wholemount preparation (figure 4b-b''), the antibody CD133, also known as prominin-1 in rodents and humans, was used to visualize NSCs from the *en face* view. This protein has also been associated with many other stem cells throughout the body, cancerous cells, as well as NSCs (Wu and Wu, 2009). Beta-catenin and gamma-tubulin respectfully outlined the ventricular surface locations of cellular junctions and the cilia of ventricular cells, predominantly ependymal cells. In both cases, structures were co-labeled for the NSC and non-NSC markers as indicated by the thick arrows in figure 4 highlighting how NSC markers are not exclusive to NSCs.

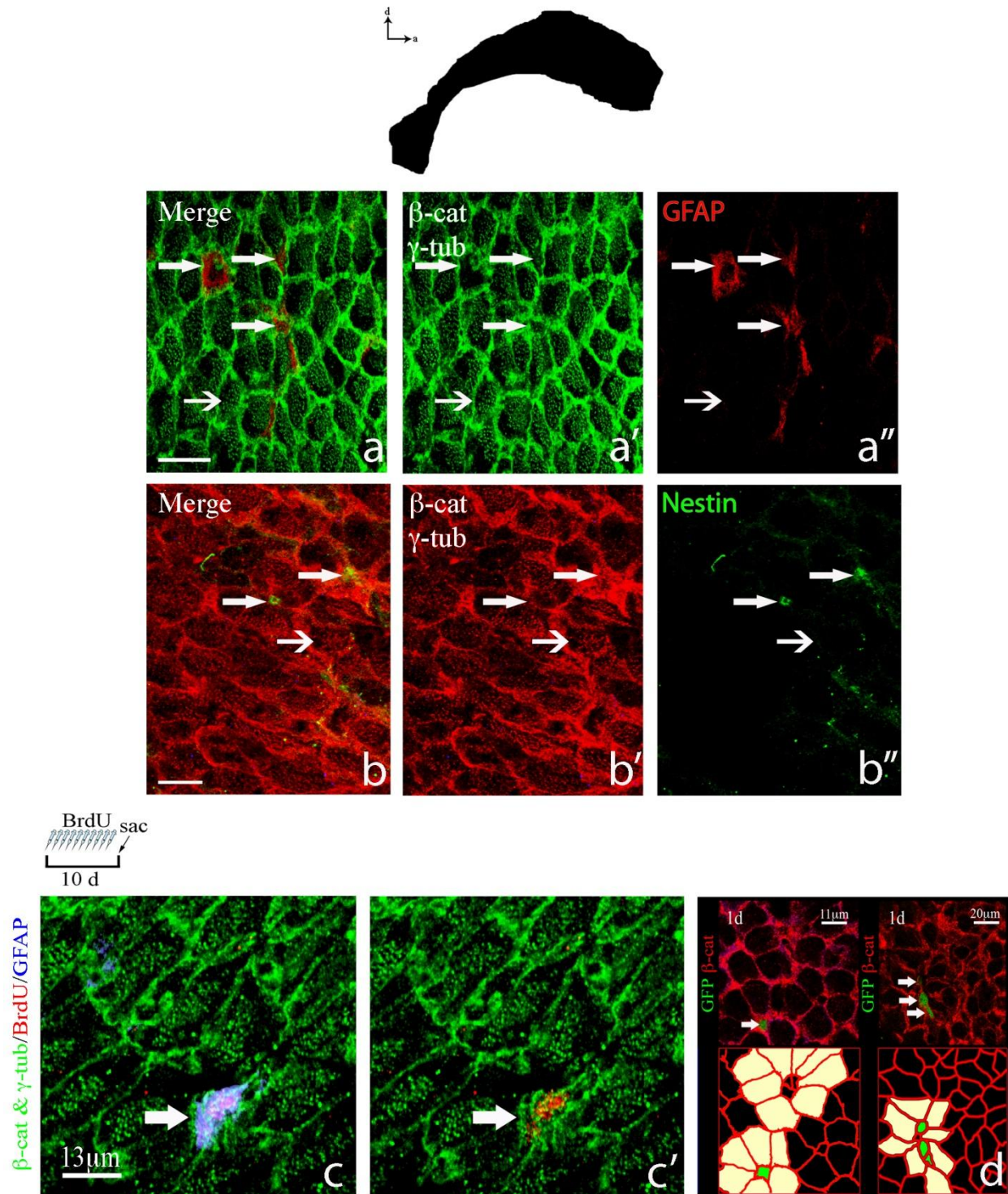


**Figure 4 Non-specificity of NSC Markers.** (a-a'') Coronal sections of the young adult 3mo SVZ were immunostained for Sox2, a NSC marker, and S100B, a non-NSC marker. Some cells did only express Sox2 (thin arrow). However, many of the cells within the region were co-labeled for both markers (thick arrow). (b-b'') Wholemount preparations of the young adult (3mo) SVZ were taken of the adult SVZ and immunostained for CD133, a NSC marker, and beta-catenin and gamma-tubulin, non-NSC markers. Many of the ependymal cells outlined by beta-catenin and whose cilia were labeled with gamma-tubulin also had cilia co-labeled with the NSC marker CD133. LW, lateral wall. MW, medial wall.

## B. Ventricle Contacting Astrocytes are Neural Stem Cells

To verify that ventricle contacting astrocytes are NSCs, wholemount preparations, revealing an *en face* view of the ventricular surface, were immunostained with the NSC marker nestin and the astrocytic marker GFAP along with beta-catenin and gamma-tubulin to again outline cellular junctions and cilia of the ventricular surface. As is evident with figure 5a (thick arrows), GFAP+ astrocytes contact the ventricular surface. Moreover, the nestin staining reveals that NSCs contact the ventricular surface as well. For figures 5c and 5c', a series of BrdU injections were given to mice for 10 days. After this

period, the brains were harvested and wholemount preparations were stained for beta-catenin and gamma-tubulin to delineate the ventricular surface. The preparations were also immunostained for GFAP. The BrdU+ nuclei of proliferating cells co-labeled with GFAP indicating that the astrocytes contacting the ventricular surface proliferate (for an example see figures 5c-c'). To further identify mitotically active cells of the ventricular surface the Lazarus (LZRS) retrovirus was injected into the ventricle. Once injected into the ventricle, the virus was taken up by dividing cells of the ventricular surface. Following infection by the retrovirus, it was shown that GFP+ cell clusters (figure 5d thick arrows) were surrounded by ependymal cells in a pinwheel structure.



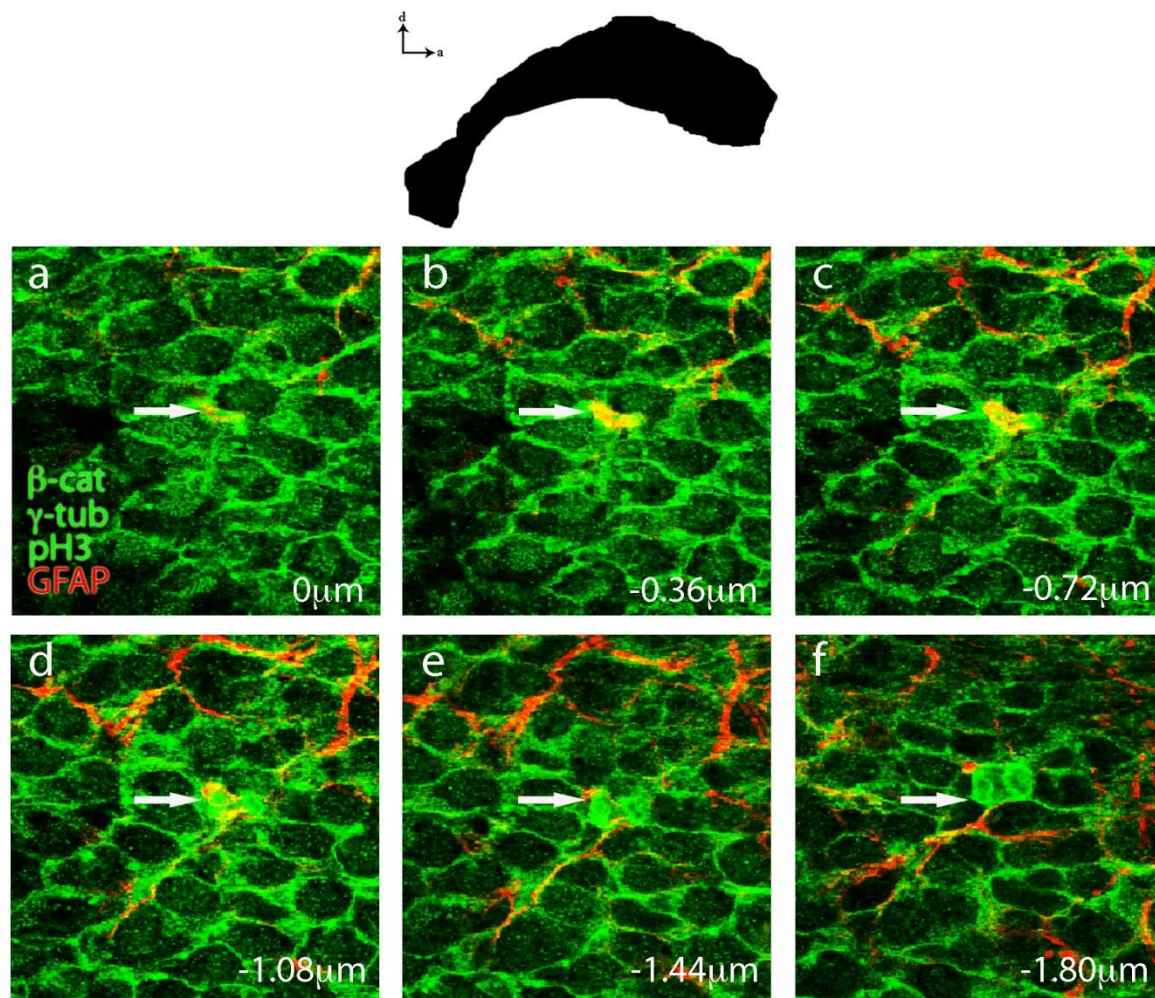
**Figure 5 Ventricule Contacting Astrocytes are NSCs.** (a-a'') Wholemount preparations of the young adult (3mo) SVZ were immunostained with beta-catenin and gamma-tubulin to distinguish GFAP- endymal cells (thin arrows) from GFAP+ astrocytes contacting the ventricular surface (thick arrows). Nestin, a marker typically used for NSCs was also found on the ventricular surface of the SVZ. (c-c') Note how BrdU, labeling proliferating cells, co-labels with GFAP (thick arrow), the astrocytic marker, and not an



ependymal cell at 3-months-old. (d) Following LZRS retroviral infection in 3-month-old mice brains, mitotically active GFP+ cells contacting the ventricle and were surrounded by ependymal cells in a pinwheel structure. The amount of astrocytic processes found in the center of these pinwheels varied. Both raw images as well as schematic images are displayed here.

### C. Detection of Neural Stem Cells for pH3

To study the proliferative NSC pool, wholemount preparations stained to delineate the ventricular surface using beta-catenin and gamma-tubulin. GFAP labeled astrocytic processes and molecular markers pH3, Ki67, and MCM2 were used to label the nuclei of proliferative cells. Using the software, Stereo Investigator (Microbrightfield, Inc, Williston, VT) a GFAP+ processes were traced down to the proliferative nuclei within the subventricular zone. Cells whose GFAP+ processes were able to be traced down to proliferative nuclei were considered proliferative stem cells for this study.



**Figure 6 Detection of GFAP+/pH3+ NSC at 3-months-old.** (a) Note how the GFAP+ process is present at the ventricular surface (thick arrow). (a-f) Also note how this process co-labels with the proliferative nucleus of the ventricle contacting astrocyte (f) The nucleus is fully visualized 1.80µm from the ependyma, which is consistent with previous studies visualizing apical, ventricle-contacting astrocytes within 5µm of the ependyma (Shen *et al.* 2008).

## V. DISCUSSION

The technique of using solely molecular markers to prospectively identify SVZ NSCs has proven to produce inconclusive results in the field neural stem cell biology (Conover and Shook, 2010). This study demonstrated how non-exclusive NSC molecular markers can be when cells of the SVZ were co-labeled for both NSC and non-NSC markers (figure 4). In the coronal section using Sox2 as a NSC marker (figure 4a-a'') very little, if anything at all, distinguished NSCs from ependymal cells. Sox2 stained both ependymal cells (figure 4a, thick arrow) and possible NSCs (figure 4a, thin arrow). This pattern also repeats in the wholemount preparation (figure 4b-b'') as the cilia of both ependymal cells and ventricle-contacting astrocytes are labeled. Due to the possible inaccuracies from using markers alone, this study has utilized both molecular markers and the morphology of ventricle-contacting astrocytic NSCs (from apical process to nucleus) in order to properly identify SVZ NSCs (figures 5 and 6).

Ventricle-contacting astrocytes being the NSCs is an idea that has been around since the late 1990s-early 2000s (Doetsch *et al.*, 1997, 1999b; Conover *et al.*, 2000); and the current study will use this morphology as a new criterion towards definitively identifying SVZ NSCs. With immunostained wholemounts, the study was able to show that GFAP+ cells, astrocytes, contact the ventricle (figure 5a-a''). Moreover, nestin+ cells, a marker widely used to label NSCs, were also evident at the ventricular surface (figure 5b-b''). Not only were these ventricle-contacting astrocytic NSCs present, the study also provides evidence that these cells are mitotically active as they were co-labeled with GFAP and BrdU (figure 5c-c'); and this study demonstrated that these ventricle-contacting astrocytes were able to take up the LZRS retrovirus (figure 5d), which is only taken up in cells where the nuclear membrane breaks down—the case in cells undergoing mitosis, NSCs, and not ependymal cells.

As a result of improper identification of NSCs, the field still remains uncertain about how the proliferative activity of NSCs cells changes with age. In other systems throughout the body, the stem cells of the region are usually identified by various positive and negative markers as well as morphological changes to the cell and/or its niche. Therefore, to localize SVZ NSCs using both molecular markers and morphology places the field of neural stem cell biology in line with other human biological systems and ahead of any published study in the field.

In order to attain results regarding the division kinetics of NSCs, this study is currently examining proliferating SVZ NSCs at certain time points (3mo, 6mo, 1yr, 2yr). Observing this active fraction will help to determine how alterations with these NSCs contribute to neurogenesis. In the case that there is a decline in the proliferative NSC population, we can see how this decline parallels the reduced neurogenesis witnessed throughout aging implying that when there is a reduced number of NSCs there are going to be less progenitor cells. With an increase in the proliferating NSC fraction, the SVZ niche may be attempting to compensate itself for a decreased ability in NSCs, or their progeny, to properly proliferate and differentiate that may factor into the decrease in neurogenesis during aging. The system may also be trying to compensate for the aging niche that has a reduced presence of growth factors. There is also the prospect that the percentage of proliferating NSCs stays the same. If this is the case the following instances tie this option to lessened neurogenesis: the intrinsic ability of NSCs or their progeny to proliferate and differentiate may have been compromised with age; the niche may be inhibiting the proliferative capacity of these cells; or the NSCs are engaged in both neurogenesis and ependymal cell replacement, alluding to the bi-potentiality of NSCs with age (Luo *et al.*, 2006).

Although data regarding the trends in the proliferative fraction of SVZ NSCs throughout aging could not be provided in this study, our laboratory does have data noting a decline in the amount of pinwheel structures throughout aging (Sane and Shook, unpublished data) which may correlate to a possible reduction in the amount of proliferative NSCs in the region.



As inferred earlier, a possible cause of the reduction in neurogenesis throughout aging may result from dysfunctional, aging NSCs. Although stem cells are critical to the regeneration of multiple tissue types for the lifetime of an organism and hence must be well preserved; these cells can become faulty with age as other cells throughout a biological system (Chamber *et al.*, 2007). With repetitive cellular division, there is repetitive DNA replication. Telomeres located at the ends of the chromosome prevent chromosome shortening and thus prevent the degradation of the coding portions of the genome. Telomerase is an enzyme that lengthens the telomeres in cells that regularly proceed through the cell cycle (Sahin *et al.*, 2010). As the SVZ maintains the largest capacity for neurogenesis, the highest levels of telomerase are expressed in this region compared to other regions of the brain (Caporaso *et al.*, 2003). Nevertheless, a waning of telomerase activity is apparent after birth (Caporaso *et al.*, 2003). With such decreases, the probability of genomic anomalies within these cells increases which may strip the stem cells ability to self-renew or differentiate properly. Stem cells must be adept to deal with environmental and internal stresses. When genes that aid in the response to stress are conditionally knock-out (such as FOXO transcription factors; ATR—ataxia telangiectasia and Rad3 related; and ATM—ataxia telangiectasia mutated) mice stem cells are unable to defend against a variety of stress and cytoarchitectural changes (Sahin *et al.*, 2010).

It is very rational to suppose that decreased neurogenesis with aging has several culprits working in conjunction with one another including: an aging niche where there is a decrease in growth factors and other mitogens (Ahlenius *et al.*, 2009), an aging NSC, declines in the amount of available or active NSCs, and the bi-potential role of NSCs in neurogenesis and ependymal cell replacement acquired with age.

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